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## Characterization of Murine Gammaherpesvirus 68 Glycoprotein B (gB) Homolog: Similarity to Epstein-Barr Virus gB (gp110)

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**Murine gammaherpesvirus 68 (MHV-68) is a natural pathogen of murid rodents and displays similar pathobiological characteristics to those of the human gammaherpesvirus Epstein-Barr virus (EBV). However, in contrast to EBV, MHV-68 will replicate in epithelial cells in vitro. It has therefore been proposed that MHV-68 may be of use as a model for the study of gammaherpesviruses, EBV in particular, both in vitro and in vivo. The EBV homolog of herpes simplex virus glycoprotein B (gB), termed gp110, is somewhat unusual compared with those of many other herpesviruses. We therefore decided to characterize the homolog of gB encoded by MHV-68 (termed MHV gB) to observe the properties of a gammaherpesvirus gB produced in epithelial cells and also to test the relatedness of MHV-68 and EBV. The MHV gB-coding sequence was determined from cloned DNA. The predicted amino acid sequence shared closest homology with gammaherpesvirus gB homologs. Biochemical analysis showed that MHV gB was a glycoprotein with a molecular weight of 105,000. However, the glycans were of the N-linked, high-mannose type, indicating retention in the endoplasmic reticulum. In line with this, MHV gB was localized to the cytoplasm and nuclear margins of infected cells but was not detected on the cell surface or in virions. Additionally, anti-MHV gB antisera were nonneutralizing. Thus, the MHV gB was unlike many other herpesvirus gBs but was extremely similar to the EBV gB. This highlights the close relationship between MHV-68 and EBV and underlines the potential of MHV-68 as a model for EBV in epithelial cells both in vitro and in vivo.**

Murine herpesvirus 68 (MHV-68) was isolated from a free-living murid rodent (3). Genetic and biological analyses have revealed that MHV-68 is closely related to the gammaherpesviruses, in particular the Epstein-Barr virus (EBV). Thus, a limited amount of MHV-68 sequence information has revealed a number of genes with strong homology to gammaherpesvirus genes and has shown that these genes lie in a similar position in the genome to those of EBV and herpesvirus saimiri (10, 11). After initial infection in vivo, MHV-68 replicates in epithelial cells and then spreads into B lymphocytes, where the virus becomes latent and persists (35–37). This initial infection is also controlled by a CD8 T-cell response (12). In addition to its B-cell tropism in vivo, MHV-68 infects and persists in B cells in vitro, where, like EBV, the genome persists in a mixture of episomal and linear forms (37). In contrast to EBV, however, MHV-68 will readily replicate in epithelial cells in vitro and is therefore an attractive model for analyzing the molecular and biochemical properties of gammaherpesviruses in infected cells.

Glycoprotein B (gB) is the most highly conserved glycoprotein between herpesviruses, with the herpes simplex virus (HSV) gB being the prototype (30). HSV gB is a large glycoprotein which is a component of virus particles and can also be found on the nuclear, cytoplasmic, and plasma membranes of infected cells (16, 26). It has a large external domain

which is glycosylated, a transmembrane domain which acts as a membrane anchor, and a shorter cytoplasmic domain (4, 17, 25). There are two forms of HSV gB in the cell: a precursor form on which the glycosylation is immature and a fully processed form (19, 39) which is present in virus particles (28). gB is not essential for virus structure but is essential for virus penetration into the host cell (5, 18, 21, 28). In addition, it is a major immunological target for antibodies which can neutralize virus infectivity (7, 23, 29).

The gB homolog of EBV (termed EBV gB or gp110) is the only gammaherpesvirus gB studied to date. Although EBV gB shares colinear amino acid homology with HSV gB and has similar structural motifs, it has markedly different biochemical and biological properties (14, 15, 24). Thus, the glycosylation on EBV gB exists only in an immature, high-mannose form which is analogous to the precursor form of HSV gB (14). Although EBV gB can be found in the nuclear and endoplasmic reticulum (ER) membranes, it is absent from the cell surface and is not a component of the virus particle (14). As a consequence, antibodies directed against gp110 do not neutralize virus infectivity (14).

Since the EBV gB homolog is unusual among the herpesviruses, we considered that a study of MHV-68 gB (termed MHV gB) would be important to explore the relatedness of MHV-68 and EBV. In addition, since there is no easy means of studying EBV replication in epithelial cells, we considered that MHV gB would be a means of studying the properties of a gammaherpesvirus gB in epithelial cells. In this paper we describe the sequencing and characterization of the gB homolog encoded by MHV-68 and show that it is extremely similar to EBV gB (gp110).

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## MATERIALS AND METHODS

**Virus and cells.** Working stocks of MHV-68 clone g2.4 were prepared in BHK-21 cells as described by Efsthathiou et al. (11). Purified virus was obtained by the use of discontinuous Ficoll gradients as described by Blaskovic et al. (2). C127 (ATCC CRL 1616) is a mouse epithelial cell line which was derived from a mammary carcinoma (22) and was grown in Dulbecco's modified Eagle's medium (Gibco/BRL, Paisley, United Kingdom) containing 10% fetal calf serum (GlobePharm, Esher, United Kingdom). The herpesvirus DNA replication inhibitors phosphonoacetic acid and acyclovir were used as indicated at concentrations of 100 µg/ml and 100 µM, respectively.

**DNA cloning and sequencing.** All cloning techniques were performed as described by Sambrook et al. (27). The MHV-68 *Bam*HI O fragment was obtained from a genomic library prepared from virion DNA in the phagemid vector pBluescript KS(−) (Stratagene). The MHV-68 *Hind*III N fragment was obtained from a pUC13 library described previously (11). Small-scale plasmid DNA preparations were made by using Wizard Minipreps (Promega), and double-stranded templates were sequenced by using Sequenase v2.0 (U.S. Biochemicals). Overlapping sequence of the complete open reading frame was obtained on both strands by the use of oligonucleotide primers. The sequence was assembled and analyzed by using the Genetics Computer Group suite of computer programs (9).

**Northern (RNA) blot analysis.** Total cytoplasmic RNA was extracted and analyzed exactly as described previously (32). The probe used for MHV gB mRNA was the 707-bp *Bam*HI-*Hinc*II subfragment of the *Bam*HI O fragment (see Fig. 1) corresponding to nucleotides 971 to 1678 of the sequence (see Fig. 2). Molecular weight determinations were made in relation to an RNA ladder (Gibco/BRL).

**Antisera.** Monospecific anti-MHV gB immunoglobulin G (IgG) was generated as follows. The same 707-bp *Bam*HI-*Hinc*II segment of the gB open reading frame used above for Northern analysis and corresponding to gB amino acids 235 to 470 was cloned into the bacterial expression vector pGEX-1N (Pharmacia). A glutathione-S-transferase-gB fusion protein with a molecular weight of 50,000 was expressed in *Escherichia coli* and was purified on glutathione-Sepharose beads as specified by the manufacturer. Purified fusion protein was then used to immunize rabbits in combination with Hunter's Titermax adjuvant (CytRX, Norcross, Ga.). Serum was prepared from bleeds taken before and after immunization. IgG was purified from sera by using a protein G-Sepharose column (Pharmacia).

Rabbit anti-MHV-68 antiserum was prepared by using MHV-68-infected rabbit kidney cells as described previously (35).

**Western immunoblot analysis.** Cell and virus extracts ( $10^5$  cells per track or equivalent) were solubilized in sample buffer, electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (7.5% polyacrylamide), transferred to Immobilon-P membranes (Millipore), and probed with antisera as described previously (33). Purified anti-gB or preimmune IgG was used at a concentration of 4 µg/ml, and anti-MHV-68 antiserum was used at a 1:500 dilution. Reactivity was detected by using alkaline phosphatase-conjugated swine anti-rabbit IgG (Dakopatts) at a dilution of 1:1000 and developed by using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma fast; Sigma).

**Immunofluorescence analysis.** Cells were grown and infected on microscope slides and either fixed in acetone for 3 min at  $-70^{\circ}\text{C}$  before being stained or stained live without

fixation but with the addition of azide throughout the staining procedure. The staining procedures were as described previously (31, 32). Primary antibodies were used at a concentration of 20 µg/ml for purified antibodies and 1:250 for anti-MHV-68 antibodies. Reactivity was detected by using fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (Dakopatts) at a dilution of 1:30.

**Radioimmunoprecipitation.** Cells were metabolically labeled with [ $^{35}\text{S}$ ]cysteine, and proteins were immunoprecipitated and analyzed by SDS-PAGE (7.5% polyacrylamide) exactly as described previously (34). Purified IgG was used for immunoprecipitation at a concentration of 20 µg/ml. Endoglycosidase H (endo H) digestion was performed with 3 mU of recombinant enzyme (Boehringer) under the conditions described by Gong and Kieff (14).

**Virus neutralization assay.** Serum dilutions varying between 1:10 and 1:320 were added to 100 PFU of MHV-68. In parallel experiments, 5% rabbit complement (Cederlane) was added after 30 min. The virus-serum combination was then used to infect subconfluent C127 cells. After 5 days the monolayers were fixed and plaques were counted. Each serum dilution was assayed in quadruplicate on three separate occasions.

**Nucleotide sequence accession number.** The nucleotide sequence of MHV gB has been submitted to the GenBank database and has been assigned accession number U08990.

## RESULTS

**Sequence of the MHV gB homolog.** The location of the gB homolog within the MHV-68 genome and a partial restriction map of the *Hind*III N fragment are shown in Fig. 1. The gB homolog was identified from a DNA sequence obtained from the end of the *Bam*HI O fragment which showed similarity with other herpesvirus gBs. This coding sequence was also in a similar genomic position and ran in a similar direction to the gB homolog genes of the gammaherpesviruses EBV and herpesvirus saimiri (1, 24). From these data, it was predicted that the complete open reading frame would be contained within the *Hind*III N fragment. This fragment was therefore subcloned by using a combination of the enzymes *Hind*III, *Bam*HI, and *Hinc*II, and the sequence of the gene was completed by using these subclones and gene-specific oligonucleotide primers.

The DNA sequence of the gene, the predicted amino acid sequence, and the sites of consensus motifs are shown in Fig. 2. To the 5' end of the open reading frame there was a TATA box which could act as a promoter element for the gene. It is of interest that this sequence, although unusual for a TATA box, is exactly the same in both EBV and herpesvirus saimiri. To the 3' end of the gene there is a consensus polyadenylation signal sequence. By analogy with EBV, it was predicted that this was the complete transcription unit and would produce an mRNA of 2.8 kb.

Computer-assisted analysis (Table 1) showed that the predicted amino acid sequence of the complete open reading frame had high homology with other herpesvirus gBs but in particular with those of the gamma subgroup. Translation of mRNAs usually occurs at the first available ATG codon. However, analysis of the sequence around the first ATG (nucleotide 269) by the method of Cavener and Ray (6) indicated that this codon was in an extremely unfavorable context for translational initiation. In contrast, the second ATG (nucleotide 290) was in a favorable context. It would therefore be predicted that translation would start at the latter codon (amino acid 8), giving a potential precursor protein of 841 residues. Computer-assisted analysis of the predicted

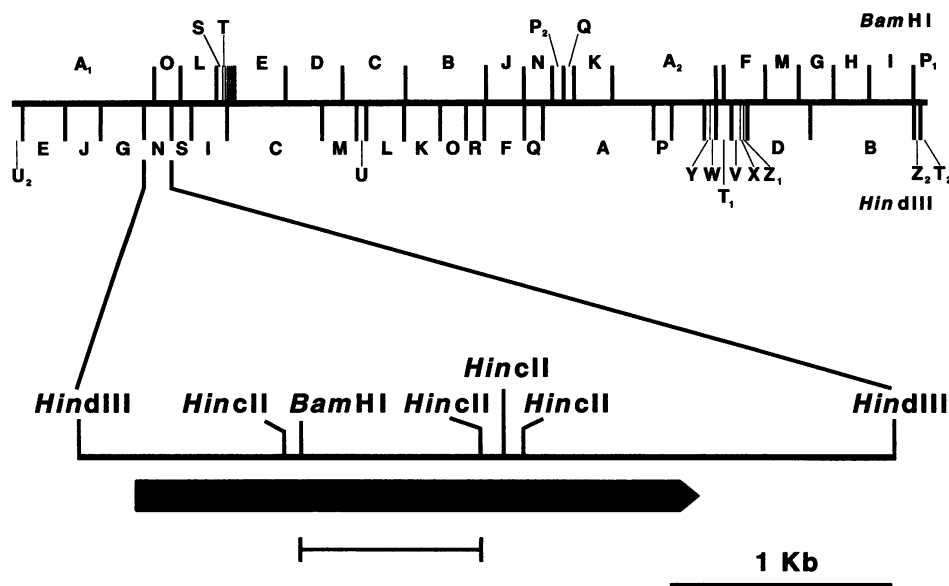


FIG. 1. Location and partial restriction enzyme map of MHV gB. The upper part of the figure is a restriction map of the unique portions of the MHV-68 genome after digestion with *Bam*HI and *Hind*III as determined by Efstathiou et al. (11). Below this is a map of the *Hind*III fragment which contains the gB open reading frame. The restriction sites shown are those which were used to subclone the fragment for sequencing purposes. The solid bar below the map represents the extent and direction of the gB open reading frame. The line below this indicates the section of the gene used as a probe on Northern blots and for expression in bacteria. A scale bar for the lower map is shown at the bottom right.

amino acid sequence by using the algorithms of Engelman et al. (13) revealed two potential hydrophobic transmembrane domains. The first of these (residues 8 to 29) displayed motifs consistent with its being a signal peptide. The position of a potential signal peptidase cleavage site, present at the end of the underlined sequence in Fig. 2, was determined by the method of von Heijne (38). Cleavage of the signal peptide would yield a potential mature core protein of 821 residues and with a molecular weight of 93,500. The second hydrophobic domain (residues 709 to 747) was longer than required for a transmembrane domain. However, it has been shown that only a small portion of the potential transmembrane domain of HSV gB acts as the membrane anchor (26). It would therefore be predicted, by analogy with HSV, that the equivalent hydrophobic domain in the MHV gB homolog would span the membrane once and act as an anchor region. Situated between these two hydrophobic domains were nine potential sites for the addition of N-linked glycosylation.

These data indicate that MHV-68 encodes a gene which is highly homologous to herpesvirus gBs, in particular those of gammaherpesviruses, not only in sequence but also in genome location and putative structural motifs. As a consequence, the protein will be referred to below as MHV gB.

**Expression of MHV gB mRNA by MHV-68-infected cells.** A primary site of MHV-68 productive replication *in vivo* is epithelial cells. Therefore, to make these and subsequent analyses as authentic as possible, we performed experiments with mouse epithelial C127 cells. Since there were no pub-

lished data on the time course of MHV-68 gene expression, cells were infected at high multiplicity of infection (10 PFU per cell) and harvested for RNA extraction at various times postinfection. In addition, infection was performed in the presence of the herpesvirus DNA replication inhibitors phosphonoacetic acid and acyclovir. Extracted RNA was analyzed by Northern blotting with the 707-bp *Bam*HI-*Hinc*II fragment (Fig. 1) of the MHV gB gene as a probe. The 28S rRNA species was used as a control for RNA loading and integrity.

Figure 3 shows that a major gB-specific RNA of 2.8 kb appeared at 8 h postinfection and was still present at 24 h postinfection. This RNA was the correct size for the complete gB transcription unit as predicted from analysis of the DNA sequence. At 24 h postinfection an additional gB transcript of 8.6 kb was observed. Both transcripts were late viral RNAs, since their production was blocked by the addition of phosphonoacetic acid or acyclovir during infection.

**Expression of MHV gB.** To analyze MHV gB, we first raised monospecific antibodies in rabbits by using a portion of gB fused to glutathione-S-transferase as the antigen, as described in Materials and Methods.

As for transcription, there were no published data on the time course of expression of MHV-68 proteins, and so C127 cells were infected as above and harvested at various times postinfection, and the expression of MHV gB was analyzed by Western blotting with monospecific anti-MHV gB IgG as a probe. The results (Fig. 4) showed that anti-MHV gB antibodies recognized a single protein with a molecular weight of

FIG. 2. Sequence of the MHV gB open reading frame. The figure shows the sequence of part of the *Hind*III fragment which contains the gB open reading frame. The DNA sequence is numbered from the upstream *Hind*III site which is the boundary between the *Hind*III G and N fragments (Fig. 1). The predicted amino acid sequence is shown above this. The sites of the consensus promoter and polyadenylation signal sequences upstream and downstream of the open reading frame are shown in boldface type and are underlined. It was predicted that the putative first 7 amino acids would not be translated, and so these are shown in lowercase type. The extents of the two hydrophobic domains are shown by underlining, and the sites of potential N-linked glycosylation are shown in boldface type and by a triangle above the residues concerned.

m y p t

180 ATATCAAAAAATCAATTGAGGGTATTTAAAGACATCTATACAATGTTAAATTTACTCTGTACATTTGAACCGATTTCAGCCAGTGTGGATGTACCCATC

5 v k s M R V A H L T N L L T L L C L L C H T H L Y V C Q P T T L R

280 AGTGAAGAGTATGAGAGTCGCCACCTAACCAATCTCCTAACCCCTCTGTGTCTGTGCCACACGCATCTCTACGTATGTCAGCCCAACCACTCTGAGG

38 Q P S D M T P A Q D A P T E T P P P L S T N T N R G F E Y F R V C G

380 CAGCCATCAGACATGACCCACGCCAGGACGCTCCAACAGAGACTCCCCACCCCTCTCAACTAACACTAACAGAGGATTTGAGTACTTTCGCGTGTGTG

72 V A A T G E T F R F D L D K T C P S T Q D K K H V E G I L L V Y K

480 GGGTGGCTGCCACGGGGAGACCTTCAGGTTTGATTTAGACAAAACATGCCCGAGTACACAAGATAAGAAGCATGTGGAGGGCATCTTGCTCGTGTATAA

105 I N I V P Y I F K I R R Y R K I I T Q L T I W R G L T T S S V T G

580 GATCAACATCGTGCCTACATCTTCAAAATCAGGAGATATAGAAAAATAATTACTCAACTGACCATCTGGCGAGGCGCTAACCACTAGTTTCACTGACTGGT

138 K F E M A T Q A H E W E V G D F D S I Y Q C Y N S A T M V V N N V R

680 AAATTTGAAATGGCCACTCAGGCCACGAGTGGGAAGTGGGGGACTTTGACAGCATCTATCAGTGTCTACAATAGCGCCACCATGGTGGTAAACAACGTC

172 Q V Y V D R D G V N K T V N I R P V D G L T G N I Q R Y F S Q P T

780 GACAGGTGTATGTGACAGAGATGGGGTCAATAAACTGTGAACATAGCCCTGTTGATGGTCTAACAGGGAATATCAAAGATACCTTAGTCAGCCAC

205 L Y S E P G W M P G F Y R V R T T V N C E I V D M V A R S M D P Y

880 CCTTTATTCAGAACCTGGTTGGATGCCTGGCTTTTATCGTGTTCGAACACCCGTTAACTGTGAAATTTGTAGACATGGTGGCAGCTCCATGGATCCCTAT

238 N Y I A T A L G D S L E L S P F Q T F D N T S Q C T A P K R A D M R

980 AACTACATCGCTACCGCCCTGGGAGACAGCCTGGAGCTCTCCCCGTTTCAAACTTTGACAACACCAGCCAGTGTACTGGCGCTAAGAGAGCTGATATGA

272 V R E V K N Y K F V D Y N N R G T A P A G Q S R T F L E T P S A T

1080 GGGTCAGGAGGTCAAGAATTACAAGTTTGTAGATTATAATACAGGGGAACCTGCCCGCTGGACAAGCAGGACCTTTCTAGAGACTCCCTCTGCCAC

305 Y S W K T A T R Q T A T C D L V H W K T F P R A I Q T A H E H S Y

1180 TTACTCCTGGAAAACAGCCACGACAACTGCCACGTGGGACCTGGTGCCTGAAAACATTCCTCGGCCATCCAACTGCTCATGAACATAGCTAC

338 H F V A N E V T A T F N T P L T E V E N F T S T Y S C V S D Q I N K

1280 CATTTTGTGGCCAATGAAGTACCGCCACCTTCAATACACCCCTGACTGAGGTAGAAAATTTACCAGCAGCTATAGCTCGCTCAGTGACCCAGATCAATA

372 T I S E Y I Q K L N N S Y V A S G K T Q Y F K T D G N L Y L I W Q

1380 AGACCATCTCTGAATATATCCAAAAGTTGAACAACTCTACGTGGCCAGTGGGAAAACAGATATTTCAAGACTGATGGTAACTGTACCTCATCTGGCA

405 P L E H P E I E D I D E D S D P E P T P A P P K S T R R K R E A A

1480 ACCACTCGAACATCCAGAGATTGAAGACATAGACGAGGACAGCGCCAGAACCAACCCCGCCCAAGTCCCAAGGAGAAAAAGAGAGGACGCT

438 D N G N S T S E V S K G S E N P L I T A Q I Q F A Y D K L T T S V N

1580 GACAAATGAAACTCAACATCTGAGGTCTCAAAGGGCTCAGAAAATCCGCTCATTTACGGCCCAATTCAAATTTGCCTATGACAAGCTGACCACCAGCGTCA

472 N V L E E I S R A W C R E Q V R D T L M W Y E L S K V N P T S V M

1680 ACAACGTGCTTGAGGAGTTGTCCAGGGCTGGTGTAGAGAACAGGTGACAGACACCCCTCATGTGGTATGAGCTTAGCAAGTCAACCTACGAGTGTGAT

505 S A I Y G K P V A A R Y V G D A I S V T D C I Y V D Q S S V N I H

1780 GTCTGCCATTTATGAAAGCCTGTCGCTGCCAGGTACGTGGGCGACGCCATATCTGTGACAGACTGTATCTATGTGACCAAAGTTCACTCAACATCCAC

538 Q S L R L Q H D K T T C Y S R P R V T F K F I N S T D P L T G Q L G

1880 CAGAGCTTGCGGCTGCAGCATGATAAAACACCTGCTACTCGAGACCTAGAGTCACTTCAAATTTATAAACAGTACAGCCCGCTAACTGGCCAGTTGG

572 P R K E I I L S N T N I E T C K D E S E H Y F I V G E Y I Y Y Y K

1980 GTCCTAGAAAAGAAATATCTCTCCAACACAAACATAGAAACATGCAAGGATGAGAGTGAACACTACTTCTATTGTGGGGAATACATTTACTATTATAA

605 N Y I F E E K L N L S S I A T L D T F I A L N I S F I E N I D F K

2080 AAATTTACATTTTGAAGAAAAGCTAAACCTCTCAAGCATCGCTACCTAGACACATTTATAGCCCTCAATATCTCATTTATTGAAAATATCGACTTCAAA

638 T V E L Y S S T E R K L A S S V F D I E S M F R E Y N Y Y T Y S L A

2180 ACAGTAGAACTGTACTCCTCTACTGAAAGGAACTCGCATCGAGCGTCTTTGATATAGAATCCATGTTTAGGGAATATAACTATTACACCTACAGCCTCG

672 G I K K D I D N T I D Y N R D R L V Q D L S D M M A D L G D I G R

2280 CGGGCATTAAGAAGGACCTAGACAACACCATCGACTACAATAGAGACAGACTGGTTCAGGACCTGTGACACATGATGGCTGATCTGGGAGACATTTGGAAG

705 S V V N V V S S V V T F F S S I V T G F I K F F T N P L G G I F I

2380 ATCTGTGGTGAATGTGGTCACTCGGTAGTCACATTTTTCAGTAGTATTGTGACAGGGTTCATTAATTTCTTTACCAACCCCTTAGGGGAATATTCTATT

738 L L I I G G I I F L V V V L N R R N S Q F H D A P I K M L Y P S V E

2480 CTCTCAATTTATGGTGAATAATCTTCTTGGTGTAGTCTTAATAGAAGAACTCACAGTTTCAGATGCACCCATCAAAATGCTGTACCTTCTGTGTG

772 N Y A A R Q A P P P Y S A S P P A I D K E E I K R I L L G M H Q V

2580 AAAACTACGCTGCCAGACAGGCGCCACCTCCCTATAGCGCATCACCTCCAGCTATAGACAAGAGGAAATTAAGCGCATACTTTTGGGCATGCATCAGGT

805 H Q E E K E A Q K Q L T N S G P T L W Q K A T G F L R N R R K G Y

2680 ACACCAGGAAGAAAAGGACACAGAAACAACTAACCAACTCTGGCCCTACTTTGTGGCAGAAAGCCACAGGATTCCTTAGAAATCGCCGGAAGGATAC

838 S Q L P L E D E S T S L

2780 AGCCAATTCCTCTGGAAGATGAATCAACTTCCCTCTAGTGGCCACCCATGACTCAGACACTTTAAAACTAACTTTGAATATAAACTGGTTTCTG

TABLE 1. Comparison of gB homologs from representative herpesviruses in relation to MHV gB

Subgroup <sup>a</sup>	Virus <sup>b</sup>	No. of codons	% Identity <sup>c</sup>	% Similarity <sup>c</sup>
$\alpha$	HSV-1	904	26	46
	HSV-2	904	28	48
	VZV	868	25	46
$\beta$	CMV	906	31	50
$\gamma$	EBV	857	42	60
	HVS	808	49	67
	MHV-68	841	100	100

<sup>a</sup> Individual herpesvirus subgroups.<sup>b</sup> Abbreviations: HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus; CMV, cytomegalovirus; HVS, herpesvirus saimiri.<sup>c</sup> Amino acid sequences were compared by using the GAP option of the Genetics Computer Group software package (9) with the gap weight and length parameters set at 3.0 and 0.1, respectively. Amino acid sequence identity and similarity relative to MHV gB, based on conservative substitutions (8), are given for homologous protein sequences.

105,000 in MHV-68-infected cells. An identical blot probed with pre-immune IgG was entirely negative, and thus the antibody was specific. MHV gB was not detected in infected cells until 24 h postinfection and was still present at a slightly lower level at 48 h postinfection. Thus, the time course of MHV gB expression was consistent with but delayed relative to RNA expression, indicating that gB was produced late in the virus productive replication cycle.

**Nature of glycosylation of MHV gB.** The predicted molecu-

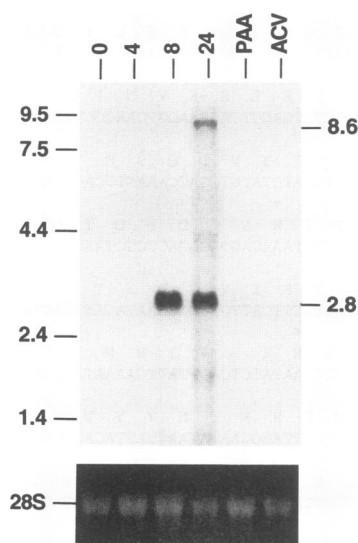


FIG. 3. Northern blot analysis of MHV gB transcription. Mouse epithelial (C127) cells were infected with 10 PFU of MHV-68 per cell and harvested at 0, 4, 8 and 24 h postinfection as indicated above the lanes. In addition, cells were infected in the presence of phosphonoacetic acid (PAA) or acyclovir (ACV) and harvested at 24 h postinfection. Cytoplasmic RNA was extracted from the cells and analyzed by Northern blotting with part of the gB open reading frame (Fig. 1) as a probe. An autoradiogram of the blot is shown in the upper panel, with the positions of an RNA size ladder (in kilobases) to the left. The sizes of the major gB-specific RNAs (in kilobases) are indicated on the right. The lower panel shows an ethidium bromide-stained image of 28S rRNA as a control for RNA loading and integrity.

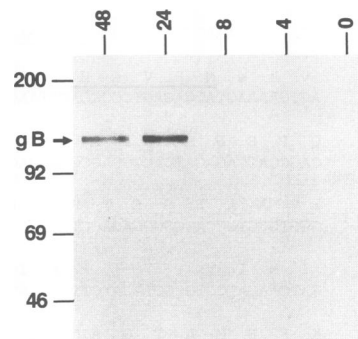


FIG. 4. Western blot analysis of MHV gB expression. Cells were infected with MHV-68 at 10 PFU per cell and then harvested at 0, 4, 8, 24, and 48 h postinfection as indicated above the lanes. Cell extracts were analyzed by Western blotting with anti-gB antibodies as a probe. The positions of protein molecular weight standards (in thousands) are shown on the left, as is the position of the gB band.

lar weight of mature core MHV gB was 93,500. Since the predicted amino acid sequence contained nine potential sites for N-linked glycosylation, it seemed likely that the protein with a molecular weight of 105,000 expressed in MHV-infected cells was a glycoprotein. In addition, like EBV gB and unlike HSV gB, which has a precursor form and a mature form with distinct molecular weights, MHV gB appeared as a single-molecular-weight species. This suggested that MHV gB might be like EBV gB and contain only immature, high-mannose, N-linked glycosylation (14).

Initial radioimmunoprecipitation studies with [<sup>14</sup>C]glucosamine-labeled, infected-cell extracts revealed that MHV gB was glycosylated (data not shown). To investigate the nature of the glycosylation, we studied the effect of endo H on MHV gB. This enzyme will digest only N-linked glycans of the high-mannose type, leaving fully processed glycosylation intact. Cells were metabolically labeled between 16 and 20 h postinfection with [<sup>35</sup>S]cysteine, a time at which MHV gB synthesis was occurring as predicted from the Northern and Western analyses. Radiolabeled proteins were immunoprecipitated with either anti-MHV gB or preimmune IgG, and a portion of the immunoprecipitate was treated with endo H.

The results (Fig. 5) showed that no labeled proteins were precipitated from either mock-infected cells with anti-MHV gB or MHV-68-infected cells with preimmune IgG. Anti-MHV gB immunoprecipitated a protein with a molecular weight of 105,000, corresponding to MHV gB, from MHV-68-infected cells. All of this protein detected was reduced in molecular weight to 92,000 after digestion with endo H. The size of the endo H-digested protein is similar to the predicted molecular weight of core MHV gB. An endo H-resistant protein with a molecular weight of 46,000 was also precipitated specifically by anti-MHV gB IgG from MHV-68-infected cells. The nature of this band is not known; however, the results of [<sup>14</sup>C]glucosamine labeling and of pulse-chase experiments revealed that it was nonglycosylated and cotranslational with (and therefore not a breakdown product of) MHV gB.

Thus, MHV gB with a molecular weight of 105,000 is a glycoprotein and resembles EBV gB (gp110) in that it contains only high-mannose carbohydrate.

**Intracellular localization of MHV gB.** Since the possession of endo H-sensitive carbohydrate is characteristic of proteins trapped in the ER (20), we next analyzed the intracellular localization of MHV gB. Cells were grown on slides and then infected with MHV-68. They were then either fixed and

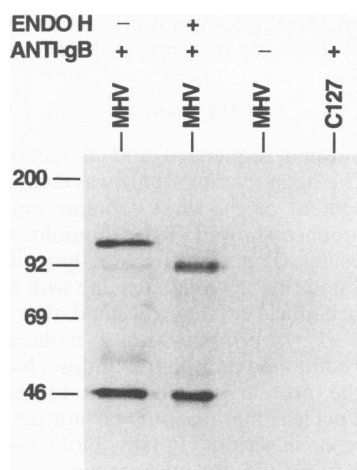


FIG. 5. Endo H sensitivity of MHV gB. Cells were either mock infected (lane labeled C127) or infected with MHV-68 at 10 PFU per cell for 16 h (lanes MHV) and then metabolically labeled with [ $^{35}$ S]cysteine. Proteins were immunoprecipitated with either preimmune IgG (indicated by -) or anti-gB IgG (indicated by +). An aliquot of the proteins immunoprecipitated by anti-gB was exposed to endo H (indicated by +). The positions of molecular weight markers (in thousands) are shown on the left.

permeabilized by using acetone prior to immunofluorescence analysis or stained live without being fixed.

The results are shown in Fig. 6. Figure 6C and E shows that there was no immunofluorescent staining after acetone fixation with either the anti-MHV gB IgG on mock-infected C127 cells or pre-immune IgG on MHV-68-infected cells. However, when anti-MHV gB IgG was reacted with acetone-fixed MHV-68-infected cells (Fig. 6A), there was intense staining which was localized to diffuse patches in the cytoplasm and in the nuclear rim. Such a staining pattern is reminiscent of proteins which are localized in the ER and nuclear envelope (17). In contrast (Fig. 6G), no immunofluorescence was seen when anti-MHV gB was reacted with live, unfixed MHV-68-infected cells. A polyclonal rabbit anti-MHV-68 antiserum, used as a positive control, showed intense surface immunofluorescence when reacted with unfixed, MHV-68-infected cells (Fig. 6I and J) but no reactivity with mock-infected cells (Fig. 6K and L).

Thus, immunoreactive MHV-68 proteins were reaching the surface of infected cells. In contrast, and in agreement with the biochemical data, MHV gB did not appear to be expressed at the infected-cell surface but instead gave a pattern of staining which was consistent with localization in the nuclear envelope and ER.

**gB is not detected in MHV-68 particles.** On the basis of both biochemical and immunofluorescence data, MHV gB was absent from the surface of infected cells. We therefore determined whether the molecule was incorporated into MHV-68

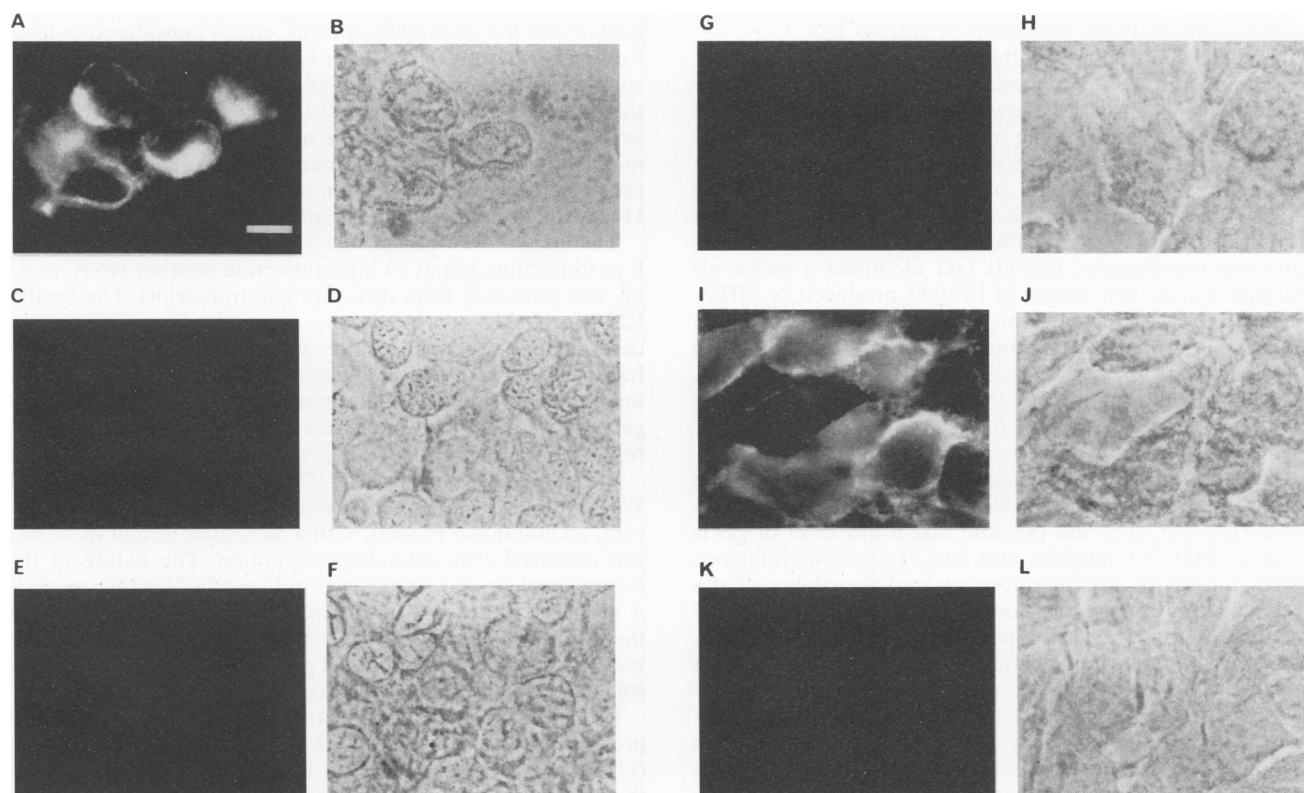


FIG. 6. Immunofluorescence analysis of MHV-68-infected cells. C127 cells were either mock infected (E, F, K, and L) or infected with 10 PFU of MHV-68 per cell for 24 h (A to D and G to J). Slides were then either fixed with acetone (A to F) or left untreated (G to L) before the immunofluorescence staining procedure. Cells were stained with either preimmune IgG (C and D), anti-gB IgG (A, B, and E to H), or anti-MHV-68 antiserum (I to L). Immunofluorescence was then visualized by using a UV microscope (A, C, E, G, I, and K). The same field, shown to the right of the UV image, was visualized by phase-contrast microscopy (B, D, F, H, J, and L). All panels are shown at the same magnification. Bar, 10  $\mu$ m.



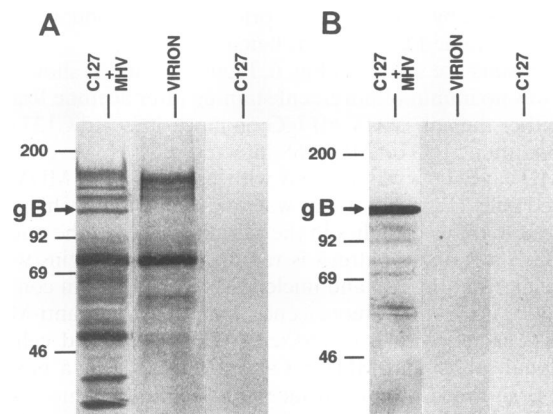


FIG. 7. Western blot analysis of MHV-68-infected cells and MHV-68 particles. Mock-infected cell extracts (lanes C127) and MHV-68-infected cell extracts (lanes C127 + MHV) were analyzed alongside an extract of purified virions from an equivalent number of cells (lanes VIRION). Identical blots were probed with either an anti-MHV-68 serum (A) or anti-gB IgG (B). The positions of molecular weight markers (in thousands) and the gB band are indicated on the left.

particles. To do this, we infected C127 cells and purified virus particles away from cellular debris. Virus particles were analyzed by Western blotting alongside MHV-68-infected and mock-infected cells. Identical blots were probed with either a polyclonal anti-MHV-68 antiserum or anti-gB IgG.

The results (Fig. 7) showed that anti-MHV antiserum identified a number of immunoreactive proteins in MHV-68-infected cells. A subset of these were also detected in MHV-68 particles, with the bands being of comparable intensity to the equivalent bands in MHV-68-infected cells, indicating the presence of similar amounts of virus proteins in the two samples. The specificity of the anti-MHV-68 antiserum was shown by its lack of reaction with mock-infected C127 cells. As in previous experiments, anti-gB IgG identified a major gB band, with a molecular weight of 105,000, produced by MHV-68-infected cells. However, this band was not detected in purified MHV-68 particles. Again, the specificity of the anti-gB IgG was shown by its lack of reaction with mock-infected cells.

Thus, these data support the thesis that, like EBV (14), the MHV gB homolog is either absent from virus particles or present at a level manyfold lower than in infected cells.

**Anti-gB antibodies do not neutralize virus infectivity.** The data presented above suggest that MHV gB is not present in virions. However, it is still possible that a low level of gB is present on MHV-68 particles and thus exerts some influence on virus infectivity. We therefore assayed the ability of the polyclonal anti-gB reagent to neutralize MHV-68. The anti-gB IgG had been raised against a portion of the molecule which, by analogy with other herpesvirus gBs (7, 23, 29), should form a significant portion of an external domain and contain neutralizing epitopes. We therefore used this reagent in a conventional plaque reduction assay with preimmune IgG as a background control and polyclonal anti-MHV-68 as a positive control. The results of three separate experiments demonstrated that anti-gB IgG exhibited no neutralizing activity over background, even in the presence of complement. In contrast, in the same experiments, the polyclonal anti-MHV-68 antiserum exhibited vigorous virus neutralization, giving >95% reduction in plaque number at all concentrations tested.

These results therefore corroborate the biochemical data

and suggest that MHV gB, even if present on the virion at low levels, does not influence the entry of MHV-68 into cells.

## DISCUSSION

We have identified, sequenced, and characterized the MHV gB homolog. The open reading frame was contained within the *Hind*III N fragment of the viral genome, and its predicted amino acid sequence showed strong homology with those of other gB molecules. Transcriptional analysis of the MHV gB gene demonstrated that it was a late gene with a major mRNA of 2.8 kb. Biochemical studies revealed that the MHV gB molecule was a glycoprotein with a molecular weight of 105,000, which contained only high-mannose, N-linked glycans. In addition, the protein was localized to the infected-cell cytoplasm and nuclear margins but was not detected either at the cell surface or in virions. Finally, biological data showed that antibodies to gB did not mediate virus neutralization.

All herpesviruses contain a homolog of the HSV gB, and it is the protein which shows most conservation among viruses. Evidence supporting the thesis that the gene sequenced here was the MHV gB homolog is as follows. First, the deduced amino acid structure had extensive homology with other gB sequences. Second, it is in a similar location in the virus genome and reads in the same direction as the EBV and herpesvirus saimiri gB open reading frames. Third, sequences from either side of the gene revealed that it is contained within a gene block conserved throughout herpesviruses. Consequently, this protein was termed MHV gB.

Although it is formally possible that there are parts of the gene which are derived by splicing, this is considered unlikely for several reasons. First, other herpesviruses gB genes are not spliced. Second, there are consensus start and stop translational signals either side of the open reading frame, which would be predicted to produce a mRNA the same size as the major transcript seen by northern blotting. Third, the size of the protein seen after digestion of the carbohydrate with endo H was the same as that of the predicted core protein.

The major MHV gB-specific 2.8-kb RNA was produced at 8 h postinfection, but at 24 h postinfection another RNA, of 8.6 kb, was observed. Both these are late transcripts. The smaller RNA probably represents the complete transcription unit described in the sequence and containing the gB open reading frame. The nature and significance of the larger transcript are unknown but indicate differences in the pattern of MHV-68 gene transcription between times around the initiation of DNA replication and at later times postinfection.

The molecular weight of MHV gB as determined by both Western analysis and immunoprecipitation was 105,000. However, an additional protein, with a molecular weight of 46,000, was observed after immunoprecipitation. The nature of this band is unclear, but since it was not seen by Western analysis, it probably represents a protein which is coprecipitated with the full-length MHV gB. Pulse-chase analysis suggested that it was not a breakdown product of gB, and thus it is likely that the smaller polypeptide is a novel cellular or viral band.

Possession of endo H-resistant carbohydrate is a feature of proteins which remain in the ER and do not reach the Golgi or cell surface (20, 26). The endo H sensitivity of MHV gB and the immunofluorescence data indicate that MHV gB is like the HSV gB precursor (19, 39) and EBV gB (14) and is present only in the ER and nuclear membranes. Thus, MHV gB must either lack a signal necessary for Golgi transport, possess its own signal for ER retention, or be targeted to the ER by a molecular chaperone. Consistent with the above is the fact that MHV gB was not detected in virus particles. It is possible that



the MHV gB was present in virions but at a considerably reduced level from that seen in infected cells, and further immunoelectron-microscopic studies are required to prove this definitively. However, if gB was present on MHV-68 particles at a low level, it did not appear to play a role in virus infection like the gBs of the alpha- and betaherpesviruses, since anti-MHV gB antibodies were nonneutralizing. Thus, like EBV gB, the function of MHV gB is unclear, but it may have some role in virus assembly or egress.

Initially, MHV-68 was classified as an alphaherpesvirus because of its ability to replicate in monolayer cultures of nonlymphoid origin (2). However, subsequent genetic and biological analyses have shown that MHV-68 is in fact more akin to the gammaherpesvirus subgroup (10, 35–37). The EBV gB (gp110), which to date is the only gammaherpesvirus gB to have been studied, is somewhat unusual compared with the gBs of both alpha- and betaherpesviruses. Our results demonstrate that MHV gB is like EBV gB in many respects. This strengthens the basis for the classification of MHV as a gammaherpesvirus and provides another demonstration of the relatedness of MHV and EBV.

There is no good system for the study of the EBV lytic cycle. In particular, there is no easy means of studying EBV replication in epithelial cells. Thus, all the studies on EBV gB have been done in B-cell lines which carry latent EBV and have been chemically induced into productive virus replication. The results obtained here with MHV and epithelial cells suggest that the discrepancies observed between EBV gB and other herpesviruses may be due to differences between herpesvirus subgroups and not to the EBV studies being performed in B cells.

Our results therefore indicate that MHV-68 may be a model for the study of various aspects of EBV and gammaherpesvirus productive replication in epithelial cells *in vitro*. In addition, MHV-68 may be a good means of studying the function in relation to the influence in pathogenesis and the immune response to gammaherpesvirus gBs *in vivo*.

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